Digestion of plasmid backbone

Thursday, 14 July 2016
3:44 PM

Worked on plasmid backbone digestion with Mariam today. Followed the digestion protocol that Mariam put on her notebook dated 14 July 2016.

We actually got stuck in the beginning because we were missing an NEBuffer2 that was needed because we needed that to use with EcoR1-HF with PST1. So we decided to ask around, and Sam from Zhao's group helped us in a few ways. Because he could not find an NEBuffer2 in their group stockpile, he lent us the HF (high fidelity https://www.neb.com/products/restriction-endonucleases/hf-nicking-master-mix-time-saver-other/high-fidelity-restriction-enzymes/high-fidelity-restriction-endonucleases) version of their PST1 so that combined with our existing EcoR1-HF, we could easily use the Cutsmart buffer. This has the added benefit that we have no star activity (https://en.wikipedia.org/wiki/Star_activity). He then showed us the NEB double digest finder (https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder) which lets us find the right buffer to use given two different restriction enzymes, so that we can search for what we need in future. But if we can begin to use HF only restriction enzymes for all our digestions, it will simplify things as they are all designed to work in Cutsmart buffer.

*KIV: Should we begin to order some HF enzymes and stockpile HF only enzymes from now on?

28 July 2016 - Transforming cells to have GFP plasmid

Thursday, 28 July 2016
12:16 PM

We used NEB C2987I high efficiency competent cells.

Vanilla protocol from NEB as follows. The SOC and pUC19 were provided as part of the NEB competent cells kit.

High Efficiency Transformation Protocol
Perform steps 1–7 in the tube provided.

1. For C2987I: Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
2. Add 1–5 µl containing 1 pg–100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4–5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.

6. Pipette 950 µl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
10. Spread 50–100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24–36 hours or 25°C for 48 hours.

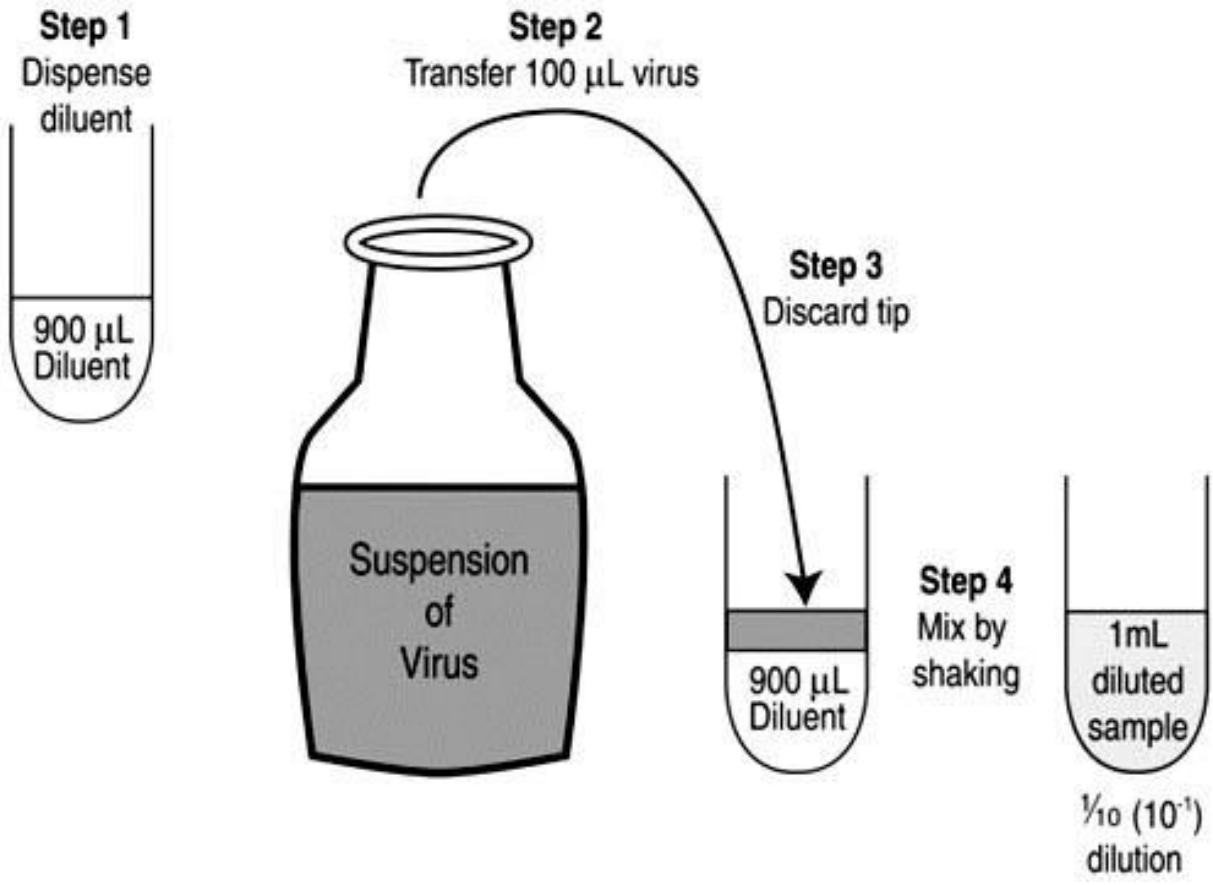
As suggested by Caroline, I set up 4 tubes. (1)Contains 1uL control pUC19 control plasmid (2) contains 1uL GFP plasmid (3) contains 2uL GFP plasmid (4)Contains no plasmid. (1) and (4) are controls. We grow (1) on plain LB, (2) and (3) on Chloramphenicol LB and (4) on Ampicillin LB (as the pUC19 plasmid has ampicillin resistance). (1) serves as a postive control, while (4) is a negative control.

I carried out steps 1-7, Viraat continued from step 7 to the end.

Some notes:
Step 7 must be while shaking. Need to cap the tubes securely, then with each containing the SOC, plasmid and competent cell mixture, place into an empty conical flask, that goes into the springy-holder in the 37degC shaking incubator. Do not incubate these in the waterbath!

INFO ADDED ON 11 August 2016

Image taken from http://www.fao.org/docrep/005/ac802e/ac802e0r.htm that describes how a single ten fold dilution is done.



Text protocol from http://www.fao.org/docrep/005/ac802e/ac802e0r.htm

Appendix 5. Ten-fold serial dilutions

A ten-fold dilution reduces the concentration of a solution or a suspension of virus by a factor of ten that is to one-tenth the original concentration. A series of ten-fold dilutions is described as ten-fold serial dilutions. In this manual, ten-fold serial dilutions are used in titrations of a suspension of Newcastle disease virus to establish the infectivity titre. They are carried out in small sterile test tubes. These tubes are usually made of glass and it is preferable if they have fitted lids to minimize the risk of contamination during the dilution.

A ten-fold dilution

Step 1. Use a micropipette to dispense 900 mL of the diluent to glass tube.

Step 2. Use a micropipette to transfer 100 mL of the test solution to the first well. Discard the tip.

Step 3. Mix by shaking by hand or using a vortex mixer.

Step 4. The well now contains 100 mL of the original test solution diluted by one tenth in a total volume of 1000 mL.

Ten-fold serial dilutions

Step 1. Set up the sterilized glass test tubes in a rack. Label each tube clearly to indicate the dilution of its contents after the ten-fold serial dilution has been carried out.

Step 2. Use a micropipette to dispense 900 mL of the diluent to all the labeled sterile tubes.

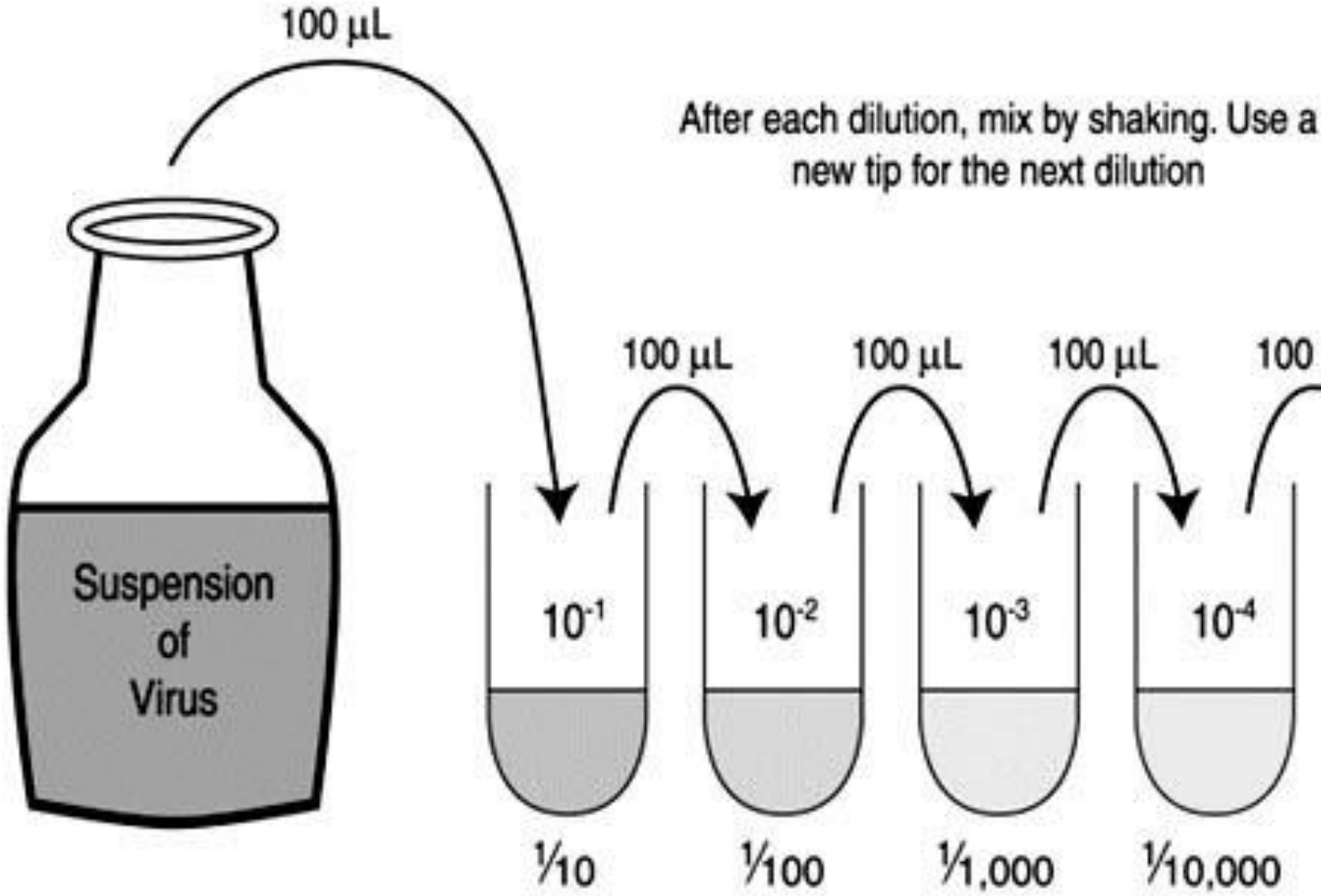
Step 3. Use a micropipette to transfer 100 mL of the test solution to the first tube and mix. This is the

first ten-fold dilution.

Step 4. Use a micropipette with new sterile tip to carry out a second ten-fold dilution.

Step 5. Continue the series of ten-fold dilutions until the last tube.

I can interpret all the amounts for my use case to be in uL instead of mL. The diluent in my case is the SOC growth mixture given by NEB.
What is meant by several 10 fold serial dilutions builds up on this concept. See the below image also taken from
http://www.fao.org/docrep/005/ac802e/ac802e0r.htm.
The picture describes it for a virus, but of course the procedure applies to us for **step 9.**



29 July 2016 - Performing miniprep on our Lycopene transformed cells (that were incubated overnight in LB tubes) with Viraat

There are two tubes, one containing BBa_K118013 and another containing BBa_K11804 that I miniprepped concurrently.

Friday, 29 July 2016
1:30 PM

Following Qiagen miniprep protocol taken from Qiagen miniprep handbook PDF file

Protocol: Plasmid DNA Purification using the QIAprep
Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on pages 35–36.

Please read “Important Notes” on pages 13–18 before starting.

Note: All protocol steps should be carried out at room temperature (15–25°C).

Procedure

1. Pellet 1-5ml of bacterial overnight culture by centrifugation at >8000rpm for 3min at room temperature

2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomesviscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

6. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.

7. Centrifuge for 30–60 s. Discard the flow-through.

8. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.

9. Wash QIAprep 2.0 spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

10. Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.
Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

11. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl ~~Buffer EB (10 mM Tris·Cl, pH 8.5)~~ or water to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min. (we used the elution buffer EB!)

Upon completion, stored the plasmids in the -20degC.

Notes as I go along the protocol

For step 1: I pipetted 1ml of solution from each glass test tube (part 118013 and 11804) into a 1.5ml centrifuge tube and the poured away the supernatant (the leftover solution). I repeated this 3 times. So in total I isolated from 3ml of solution. This was suggested by Sam Hamedi, that 3ml would be a good quantitiy.

For step 2:
Viraat came in and we continued with resuspension. Add 250uL and then suck up and push back solution multiple times to resuspend. Solution turns cloudy.

For step 3:
Yes our solution did turn cloudy after adding N3.

For step 5: The precipitate did not completely form into a white pellet but we were able to pipette out clear solution without the white stuff.

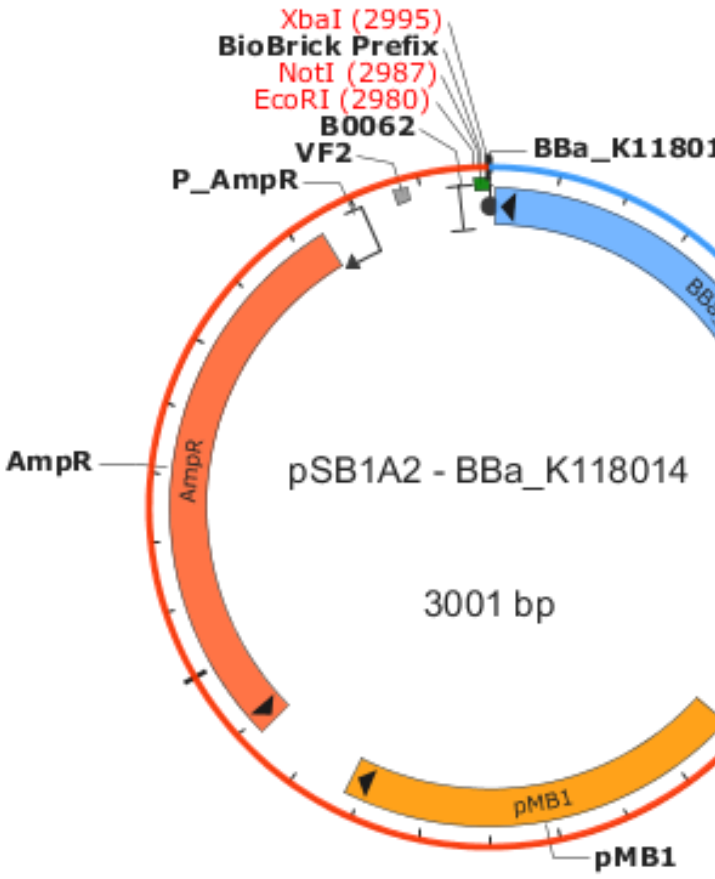
For step 8: We did not do this step as its not required for our DH5 alpha cells.

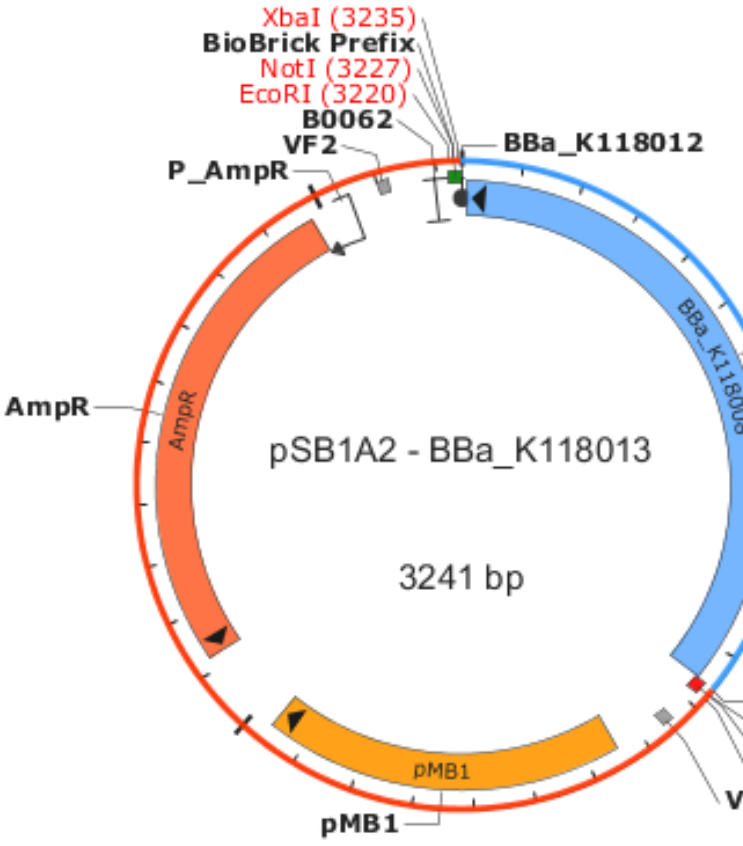
INFO ADDED ON 11 August 2016: Do not elute with buffer, elute with water, otherwise we'll have trouble when running a gel!

We could verify that our parts are correct by running a gel. First, we need to digest out the lycopene coding sequences from the plasmid backbone. In order to do digest correctly, we need to know in what plasmid backbone that the lycopene biobrick was in. Here's the details, from the email order we sent to iGEM HQ:

Part name: BBa_K118014
Plasmid backbone: pSB1A2
Sample Location: 2012 Kit Plate 2, well 18H
Quality Control Results: Sequencing confirmed
Part name: BBa_K118013
Plasmid backbone: pSB1A2
Sample Location: 2012 Kit Plate 2, well 18F
Quality Control Results: Sequencing confirmed

Procedure for digest can be found on Mariam's notebook on July 15, 2016.
I was not able to execute this as the digest required incubation time of 2hrs and would have to leave before that. But I looked up the plasmid sequence from Biobrick registry so that we'll know what to expect and they are (clicking on the View plasmid link on the pages of the respective parts):


XbaI (2995)
BioBrick Prefix
NotI (2987)
EcoRI (2980)
B0062
VF2
P_AmpR
BBa_K11801
AmpR
AmpR
pSB1A2 - BBa_K118014
3001 bp
pMB1
pMB1


XbaI (3235)
BioBrick Prefix
NotI (3227)
EcoRI (3220)
B0062
VF2
P_AmpR
BBa_K118012
AmpR
AmpR
pSB1A2 - BBa_K118013
3241 bp
pMB1
pMB1

For the Bba_K118013 http://parts.igem.org/Part:BBa_K118013 ; it contains
an RBS (Bba_K118012), and a coding sequence (Bba_K118008). Number in () in the red labels indicate the position of the respective restriction site on the plasmid.
For the Bba_K118014 http://parts.igem.org/Part:BBa_K118014 ; it contains
an RBS (Bba_K118012), and a coding sequence (BBa_I742151).
The total length of this part is 1162 bp; we shall look out for this during our gel.
The total length of this part is 922 bp; we shall look out for this during our gel.

TODO: Run the digest and gel

Experiments completed 1 August 2016

Monday, 1 August 2016
1:41 PM

Concerned that the GFP is not ok, so we need to verify it, and if we find it is not ok, then we must redo PCR for the GFP gBlocks.

Run nanodrops on the GFP stocks check concentration
Run GFP stocks on gel

If both don't show good results, (low concentration or wrong size), then repeat PCR.

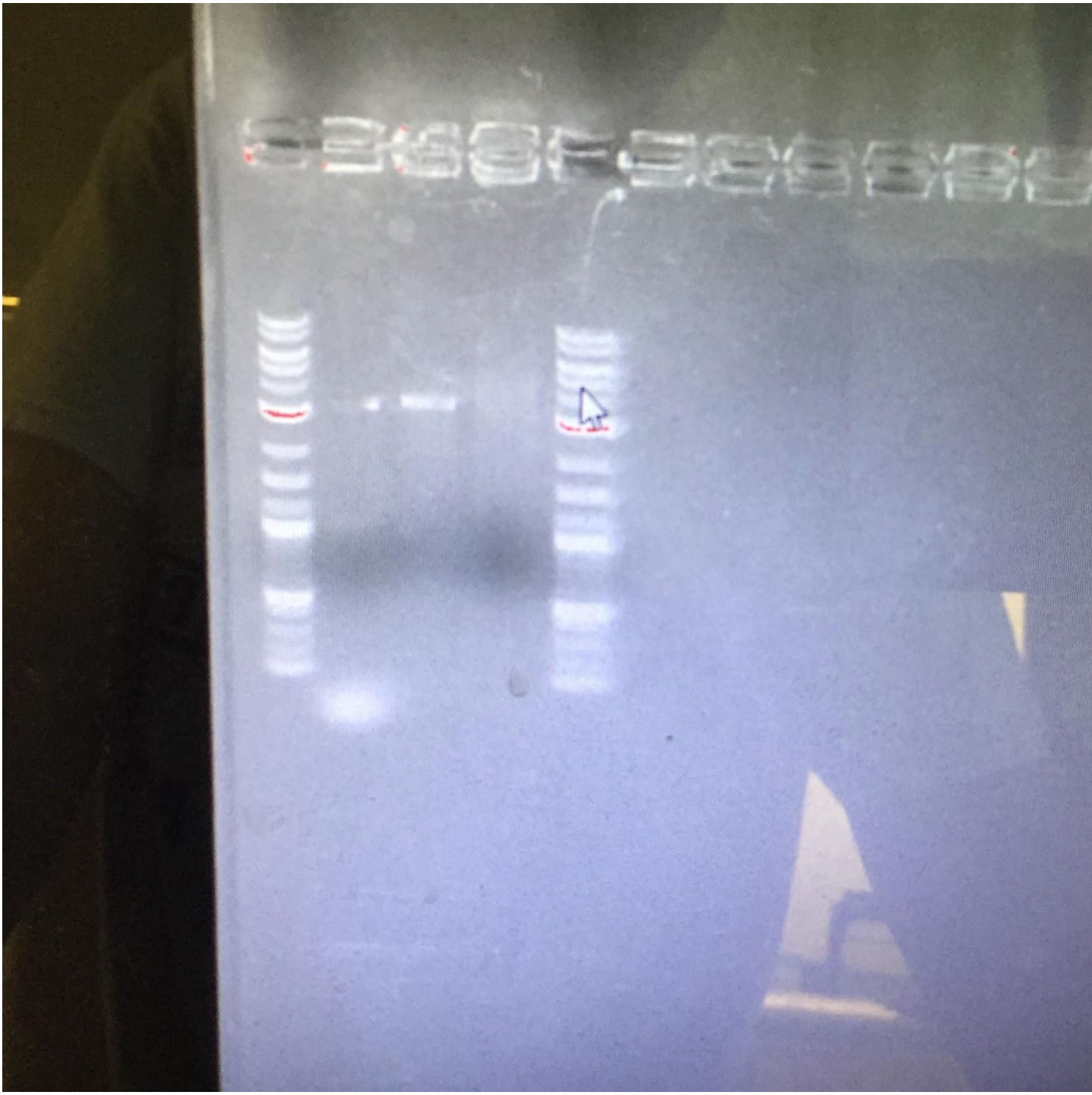
Jonathan worked on this, but because there was not much PCR product left from before, he re-did PCR.
We need to check if the competent cells Caroline made last week are ok, by transforming them with Puc19 and see whether they take up the plasmid (if they do, they will grow on ampicillin plate). This was done by Mariam, while I just did the last part of plating the cells onto an LB+Amp and putting them back into incubator.
Digest and verify Lycopene parts! Don't need to be concerned about digesting too much getting too much efficiently because all we need is some parts get digested then we can get the bands we need.

Before creating the digestion mixture, I had to measure the concentration of each plasmid stock using nanodrops. We calibrated (blanked) the machine with EB elution buffer. This is because we eluted the plasmid DNA using EB buffer on Friday. Turns out that part 118013 is at 112ng/L and part 11804 is at 107ng/L. I diluted each by 5x using ddH2O (1 part of the plasmid part in EB buffer, with 4 parts of ddH2O). I stored these in the small PCR tubes labelled with the part number and the word "diluted".

I followed Mariam's July 26th protocol for pSB1C3 digest, except that I did accidentally put 2uL of each restriction enzyme into the mixture for lycopene part 118014 (part 118013 should have correct mixture). But it should be ok, I just let the digestion run. I ran the incubation at 37degC for 30mins.

To run the gel, I only took out 6uL of the digestion product, so the remaining product are stored in tubes with part number on them and labelled "digested lyco".
NOTE: While we were trying to do our nanodrops measurements, we asked Ran Chao if we should blank our measurements with EB buffer or water, and he explained to us that if the solution holding our DNA/plasmid is an EB buffer, then use EB buffer, if water, then water. So when Viraat and I did our elution during the miniprepping on Friday, we used the Qiagen EB buffer. Ran Chao suggests that in

future, for elution, it might be better to use water. That's what they do in Zhao lab. Because if I used EB buffer, it contains a lot of minerals salts that can affect gel eletrophoresis results.



So here's our results :/
Lycoopene part 118013 is second column from the left, while part 118014 is the 3rd column from the right (excluding the DNA ladders on both left and right). So Jonathan's GFP PCR product is the first on left. All don't look good unfortunately. I wonder if for mine, it was, like what Ran Chao said, that the EB buffer interferes with the results for running gel….

2 August 2016

Tuesday, 2 August 2016
1:59 PM

In light of yesterday's failure of the lycopene run on gel, we decided to redo the miniprep, eluting with water this time (not buffer EB!). As we ran out of the lycopene cells in LB solution that we could transform directly, I had to re-incubate a new batch of lycopene cells.

Re-incubating a new batch of lycopene cells

I followed the proportions on Addgene https://www.addgene.org/mol-bio-reference/antibiotics/ , where they suggest that "…dilute your antibiotic into your LB medium at 1:1,000. For example, to make 100 mL of LB/ampicillin growth media, add 100 µL of a 100 mg/mL ampicillin stock (1000X stock) to 100 mL of LB….". I created 500mL of amp&LB solution by mixing 500uL of our ampicillin stock (which can be found in a tube in the big LB plate freezer near the gel electrophoresis bench) that is already at 100mg/mL concentration, with 500mL of plain LB solution from our lab bench. I made two of these in two separate test tubes.

Then from the two LB plates containing live colonies with the BBa_k118013 and BBa_k118014 part plasmids (which I retrieved from the same fridge holding the ampicillin solution) I scrapped off some colonies with a pipette tip and dropped the tip into the correspondingly labelled amp&LB solution test tubes. For part 118013, I did this twice, as the first time I was actually scraping much bacteria off the surface, but rather, digging too much into the gel itself.

Let this run over night. If this goes well, I should see that my solution turns cloudy.

ADDED on 3 August 2015 Apparently I realized that I made quite silly and careless mistake - I actually created only 5mL of combined amp&LB solution for each part! So that means I mixed amp and LB in an exact 1-1 ratio! But in the end, the bacteria were still able to grow and cloud the solution, so I guess that worked out well LOL

3 August 2016 - Doing miniprep again, eluting with water instead

Wednesday, 3 August 2016
1:40 PM

So here's the procedure again, this time but slightly different.

Following Qiagen miniprep protocol taken from Qiagen miniprep handbook PDF file

Protocol: Plasmid DNA Purification using the QIAprep
Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on pages 35–36.

Please read “Important Notes” on pages 13–18 before starting.

Note: All protocol steps should be carried out at room temperature (15–25°C).

Procedure

1. Pellet 1-5ml of bacterial overnight culture by centrifugation at >8000rpm for 3min at room temperature

2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

6. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.

7. Centrifuge for 30–60 s. Discard the flow-through.

8. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains
such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.

9. Wash QIAprep 2.0 spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

10. Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.
Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

11. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl ~~Buffer EB (10 mM Tris·Cl, pH 8.5) or~~ water to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min. (we used the water for elution!)

Upon completion, stored the plasmids in the -20degC.

Notes

For step 1: I transfer the LB liquid culture from glass tubes directly into large plastic centrifuge tubes and spun them in the large centrifuge at 3900rpm for 6mins.

Step 2

I just added 250uL of P1 directly into the large plastic tube and resuspended there - used the large pipette for this as it is long enough to reach in. Then after finishing resuspension, I transferred to microcentrifuge 1.5mL tube.

Completed the miniprep and eluted with water! So I stored the plasmid in tubes labelled with date on them.

DIGESTION

To carry out the digestion, again follow same protocol as Mariam's 26 July entry for pSB1C3 digestion. I measured the concentration using nanodrops/NANO QUANT PLATE, and found for part 118013, its at 141.9ng/uL while for part 118014, its at 137.5ng/uL.

I proceeded to dilute both parts by a factor of 7, by taking 2uL of each part and mixing them with 14uL of the same water in separate tubes to get a total of 20uL. So I strove to dilute both samples to 20ng/uL (an approximate value, since they are both not exactly 140ng/uL, but yes very close to that value, so I approximated to that value). Then I pipetted out 16uL of each diluted plasmid to mix with the corresponding amounts of enzymes (as detailed in Mariam's notebook). Digested for about 30mins.
Note: Forgot how to measure concentration of two liquids at the same time, so Caroline refreshed the procedure. Turns out the only difference is that instead of blanking out just one cell, I need to blank out two cells, and later use those two same cells for the measurement.

Ran on gel, with 118013 on the left column and 118014 on the right column. Here are the results.

We didn't even get any signal of the DNA… the dark diffuse bands are just the DNA gel loading dye.

So can we verify that the plasmids are lycopene? We figured that we'll just trust iGEM HQ for now that the lycopene parts are correct….

8 August 2016

Goal for lab today is to take the successfully PCRed GFP gBlock (successfully purified on Friday 4 August 2016) and assemble it to the plasmid backbone. First we must digest these parts, then ligate them.

Monday, 8 August 2016
11:12 AM

Digest

pSB1C3 backbone digest:

1. 2uL Cutsmart
2. 1uL EcoRI-HF
3. 1uL PstI-HF
4. All of our leftover linearized plasmid backbone pSB1C3 (which was barely 10uL)
5. 6uL of water

- Total reaction: 20uL, set at 20ng/uL (total volume must always be kept constant regardless of variations in the plasmid backbone amount)

Placed in 37degC incubator at 1134am

GFP digest (insert):

1. 4uL Cutsmart
2. 2uL EcoRI-HF
3. 2uL PstI-HF
4. All GFP gblock that was sucessfully PCRed out on Friday 4 August 2016 (which is about 22uL)
5. 10uL of water

- Total reaction: 40uL at about 20ng/uL

Placed in 37degC incubator at 1156am

We cannot heat kill the HF enzymes, so we just use PCR purification kit to remove them. Yes we can use PCR purification kit to clean up digestion mixture.
Follow entire procedure, except for the modification is to use 20uL of water to elute instead of elution buffer.

Took out the pSB1C3 digest at about 1234pm and performed the procedure.
Took out the GFP digest at about 108pm and performed procedure.

So I digested for about 1 hour which is about right for a diagnostic digestion (digestion just to check if our sequences of concern are of the right length (number of base pairs).

Qiagen PCR purification procedure:

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold. For details on how to set up a vacuum manifold, refer to the QIAquick Spin Handbook.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.

4. To wash, add 0.75 ml Buffer PE to the QIAquick column centrifuge for 30–60 s or apply vacuum. Discard flow-through and place the QIAquick column back in the same tube.

5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add 50 µl ~~Buffer EB (10 mM Tris·Cl, pH 8.5) or~~ water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Next step is to carry out ligation assembly. We use same old protocol, but the T4 ligase enzyme and ligase buffer is new (our old one was not working) so we need to do a mix of it. Just pipette up and down.

Can incubate at room temperature (just leave on bench). Actually no, for better results, use PCR thermocycler to set at constant 16degC overnight. How long? Just set it to be the duration from when I put it in today to 8/9am tomorrow morning.

The important thing about ligation is not so much about making sure we use the "right" amounts and the "right concentrations" but more that we use the right mass ratios and from there the mass ratios dictate the amount and concentration of the solution mixture. The mass ratio is not stated on the iGEM page though [http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones]. So I look to the AddGene page [https://www.addgene.org/plasmid-protocols/dna-ligation/] which says

#######
For most standard cloning (where the insert is smaller than the vector) a 3 insert : 1 vector ratio will work just fine. We recommend around 100ng of total DNA in a standard ligation reaction.

1. Combine the following in a PCR or Eppendorf tube:
    - 25ng Vector DNA
    - 75ng Insert DNA
    - Ligase Buffer (1µL/10µL reaction for 10X buffer, and 2µL/10µL reaction for 5X buffer)
    - 0.5-1µL T4 DNA Ligase
    - H20 to a total of 10µL

    Note: *If the DNA concentrations are low such that you cannot get all 100ng of DNA, buffer and ligase into a 10µL reaction, scale the reaction size as necessary - being sure to increase the amount of buffer proportionally. 1µL of ligase should be sufficient for larger ligation reactions.*

    Note: *Because ligase buffer contains ATP, which degrades upon freeze/thaw cycles, it is a good idea to take a fresh tube, thaw it one time and aliquot individual tubes of 5, 10 or 20µL for storage at -20°C. Whenever you need to set up ligations in the future you can thaw a new tube that you know has only been thawed once before.*

########
Note: We must always keep the total volume for each digestion constant, regardless of how much DNA part we have, because we need to ensure that the enzymes are at correct concentration.

Note for step 1: I just added 5 parts of whatever was in each tube for each part into the same tube and mixed. That was 100uL for our pSB1C3 (since we had 20uL of digestion mix), and 200uL for our GFP (since we had 40uL of its digestion mix).

Note for step 7: Do not elute with buffer! Elute with water!!!
Note for step 8: Do not need to run a gel for my individual parts, we only run gel on the assembled part.

T4 Ligase buffer can be found in 3 labelled tubes in our ligation box. Look in one of the top rows. Its at 10x concentration.

According to nanodrops measurement, we had digested GFP at 2.4ng/uL and the backbone at 3.5ng/uL. So I added (75ng)/(2.4ng/uL) = 31.25uL of GFP and (25ng)/(3.5ng/uL) = 7.14uL of backbone. I decided to scale the reaction to 50uL so I used 5uL of 10x ligase buffer, with 1uL of T4 ligase, and topped it up with 5.61uL of water for the total of 50uL.

I set this all up in the thermocycler at 16degC starting about 445pm and will be running it through to 930am tomorrow morning.

NOTE: We are running low on ligase enzyme so we may have to ask Michelle or the Zhao Group people for more.

Note: Upon thawing the T4 ligase buffer after removing it from the freezer, I forgot to mix it in place in its original tube!!!! We should always mix the buffer immediately upon thawing because a precipitate forms when it gets frozen. My reaction might not work! Argh! And I can't run another ligation set up because I've used up more than half of the 50uL of digested GFP that I generated today (if I used the reduced amounts it will be total less than 100uL and its not recommended according to the AddGene protocol).

Tomorrow I'll do another digestion of the GFP and backbone again to generate more DNA, and set up a new ligation reaction!

9 August 2016

Goal of today's experiments is to transform a batch of cells with the results of yesterday's ligation, the GFP plasmid reporter construct. At the same time, we will also transform some cells for use in the interlab study.

Then if have time, I will redo digestion of GFP and plasmid backbone (may need to regenerate a new batch of backbone if we have run out of it) to redo the ligation that I may have failed yesterday because I did not mix the ligation buffer (although I must check if anyone else can carry on this with transformation tomorrow, because I will have to be moving house and won't be able to work).

For both transformations iGEM, and our own, we use our own competent cells. They are stored in the leftmost -80 freezer of the BSD group and are nicely allocated in individual tubes of 50uL. Since each transformation of each part uses just 50uL, the number of tubes I should take out should be the same as the number of parts I need to transform. This is nice because I don't want to be taking out a large tube, thawing it, then taking out only a portion of it and freezing the leftovers. The leftover cells will not be competent anymore.

Tuesday, 9 August 2016
11:27 AM

Caroline took out the ligation mixture from the PCR thermocycler, and I heat killed the T4 enzymes still present in it by running a thermocycler run of 65degC for 10mins. Remember always to heat kill the enzymes everytime I complete a ligation!

Transformation protocol for iGEM interlab study parts (this is the generic iGEM transformation protocol, which we follow for iGEM parts)

1. Thaw competent cells on ice
   This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
2. Pipette 50µl of competent cells into 2ml tube
   50µl in a 2ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. **Don't forget a 2ml tube for your control.**
3. Pipette 1µl of resuspended DNA into 2ml tube
   Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
4. Pipette 1µl of control DNA into 2ml tube
   Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
5. Close 2ml tubes, incubate on ice for 30min
   Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
6. Heat shock tubes at 42°C for 1 min
   2ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.

7. Incubate on ice for 5min
   Return transformation tubes to ice bucket.
8. Pipette 200µl SOC media to each transformation
   SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
9. Incubate at 37°C for 2 hours, shaker or rotor recommended
10. Pipette each transformation on two petri plates for a ~~20µl and 200µl~~ 200uL plating
    Pipette 20µl and 200µl of the transformation onto appropriately labeled plates. Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
11. Incubate transformations overnight (14-18hr) at 37°C
    Incubate the plates upside down (agar side facing up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
12. Pick single colonies
    Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.
13. Count colonies for control transformation
    Count colonies on the 20µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of $1.5x10^8$ to $6x10^8$ cfu/µg DNA.

I had to resuspend the interlab study parts. There were 5 tubes with parts named
Postivie Control
Negative Control
Device 1
Device 2
Device 3

The uses of each of these devices is detailed in the plate reader protocol of the interlab study.

Like how IDT ships their DNA, iGEM ships DNA in dried form. So I centrifgued the tubes to make sure all dried DNA pieces fall to bottom. Then I added 10uL of ddH2O to resuspend the DNA (there is a total of 100picograms in each tube, so this gives a solution of 10pg/uL concentration which is what is required to match iGEM transformation protocol. I stored the leftover of these tubes into our miscellaneous DNA box.

SOC is stored in same fridge as Gel loading dye and DNA ladder

I put the tubes into the shaker incubator started at 4pm…

We are just gg to put just one plate, a 200uL one….

So I did not want to mix up between the two protocols for the two classes of transformations I am doing today, and I worked only on the iGEM one first until I started step 9 and the tubes were incubating, then moved to our own GFP construct (which needs lesser time to incubate and so I can work on following the iGEM one.
We don't need to do any controls (no need to transform any cells with a control plasmid) because we are certain that our cells are working fine.

Transformation Protocol for our own GFP construct (from Michelle, taken from Mariam's notebook and modified)

Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/

1. Take out new homemade DH5-alpha competent cells from -80C freezer and thaw on ice for about 20 min
2. Place agar plates with antibiotic in 37C incubator to warm them up.
3. In 1.5 mL microcentrifuge tube, add 50uL of competent cells.
4. Add 5uL (unless otherwise specified) of whatever plasmid or DNA (that I want to transform the cells with) to the cells in the microcentrifuge tube
5. Place microcentrifuge tube on ice for 20-30min
6. Heat shock in 42C water bath for 45 sec
7. Add 500uL of LB media liquid (freely available on our bench)
8. Place the microcentrifuge tubes into a conical flask, and secure the flask to a holder in shaking incubator at 37C for 1 hour. Make sure the tubes are capped tight.
9. Pipette out 200uL of transformation on LB agar plates
10. Pour a few beads into the plate, hold tight and shake (while holding the plate with its agar side facing down) to spread the transformation solution across entire agar.
11. Incubate in 37C overnight. Place the plates with agar side up while in incubator, to prevent any water condensing on the non agar side from dripping onto the agar.

For step 4: Also beware that too much plasmid DNA can be toxic. If using less competent cells, use less than 5uL of plasmid. I use about 4.5uL for our ligation GFP construct transform.

Following step 7: Do not subsequently place the tube on ice. Contrary to the iGEM protocol, we prefer not to place the tube on ice after we have heat shocked the cells and given them LB, because we still want them to be taking in plasmid. So for our GFP construct transformation, I did not place on ice and immediately proceeded to step 8.

At step 8: I placed our GFP construct tube in the same flask as for the iGEM interlab study tubes, at 5:01pm. When its about 1hr later at 6:01pm, I can just take out the entire flask and that will match the time needed for the iGEM parts too which is 2hrs.

At 6:01 pm, I took all the 6 tubes out of the shaker, plated them, and put them back into the incubator (top shelf - the one with the drawer. Always put agar plates in this top drawer!) for overnight growth. Note that there is one tube which did not contain 200uL (it had less than 200uL). That actually came from an earlier upstream step when the tube containing competent cell actually had less than 50uL of cells. This is for one of the iGEM interlab study parts. Shouldn't be a problems, let see how the cells grow overnight!

11 August 2016

Thursday, 11 August 2016
3:58 PM

Goals for lab today are:
1. Transform the Gibson assembled cells (that were produced on 9 August 2016) into NEB competent cells (they failed when Mariam tried it on our own homemade competent cells).

2. Prepare glycerol stocks of the two colonies of bacteria transformed with standard ligation GFP reporter construct, these two colonies grew successfully on overnight between 9-10 August, and were inoculated in liquid culture by Caroline overnight yesterday. They are labelled as colony 1 and 2. What's the difference???
3. Then miniprep these two colonies to extract the plasmid DNA

- I tried shining some UV light using the gel imager on the liquid colonies to see if they fluoresced before doing step 2 and 3. See a photo on the right.

(1) Following NEB protocol to transform Gibson DNA into NEB cells

28 July 2016 - Transforming cells to have GFP plasmid

The slight modifications to note are:
Step 2: I used 2uL of the Gibson assembled GFP construct. I HAD SUCH A HARD TIME FINDING THE TUBE CONTAINING THE CONSTRUCT! EVENTUALLY FOUND IT IN OUR PCR PRODUCTS BOX!!! **WE NEED TO FIND A BETTER WAY TO STORE THOSE TINY PCR TUBES SO THAT THEY WILL BE MUCH MORE RETRIEVABLE!**
Step 9: I diluted to two versions, one with 0-fold dilution, and another with 1-fold dilution.
Step 10: Instead of max 100uL, I spread 200uL unto of each fold of dilution unto its own plate.

When Ran Chao from Zhao Group saw that we were using those large glass test tubes for liquid culturing, he remarked that we should consider using Falcon brand 14mL Polypropylene Round -Bottom Tube 17x100mm style disposable plastic tubes. The Zhao group uses those and they have nice clip on caps.

(2) Prepare Glycerol stocks the bacteria transformed with the standard GFP ligation method (from the two colonies that were inoculated in liquid culture yesterday)

Taken from https://www.addgene.org/plasmid-protocols/create-glycerol-stock/

Protocol: Creating Bacterial Glycerol Stocks for Long-term Storage of Plasmids

1. Follow the steps for Inoculating an Overnight Liquid Culture.
2. After you have bacterial growth, add 500 µL of the overnight culture to 500µL of 50% glycerol in a 2 mL screw top tube or cryovial and gently mix.

   Note: *Make the 50% glycerol solution by diluting 100% glycerol in dH20.*

   Note: *Snap top tubes are not recommended as they can open unexpectedly at -80°C.*
3. Freeze the glycerol stock tube at -80°C. The stock is now stable for years, as long as it is kept at -80°C. Subsequent freeze and thaw cycles reduce shelf life.
4. To recover bacteria from your glycerol stock, open the tube and use a sterile loop, toothpick or pipette tip to scrape some of the frozen bacteria off of the top. Do not let the glycerol stock unthaw! Streak the bacteria onto an LB agar plate.

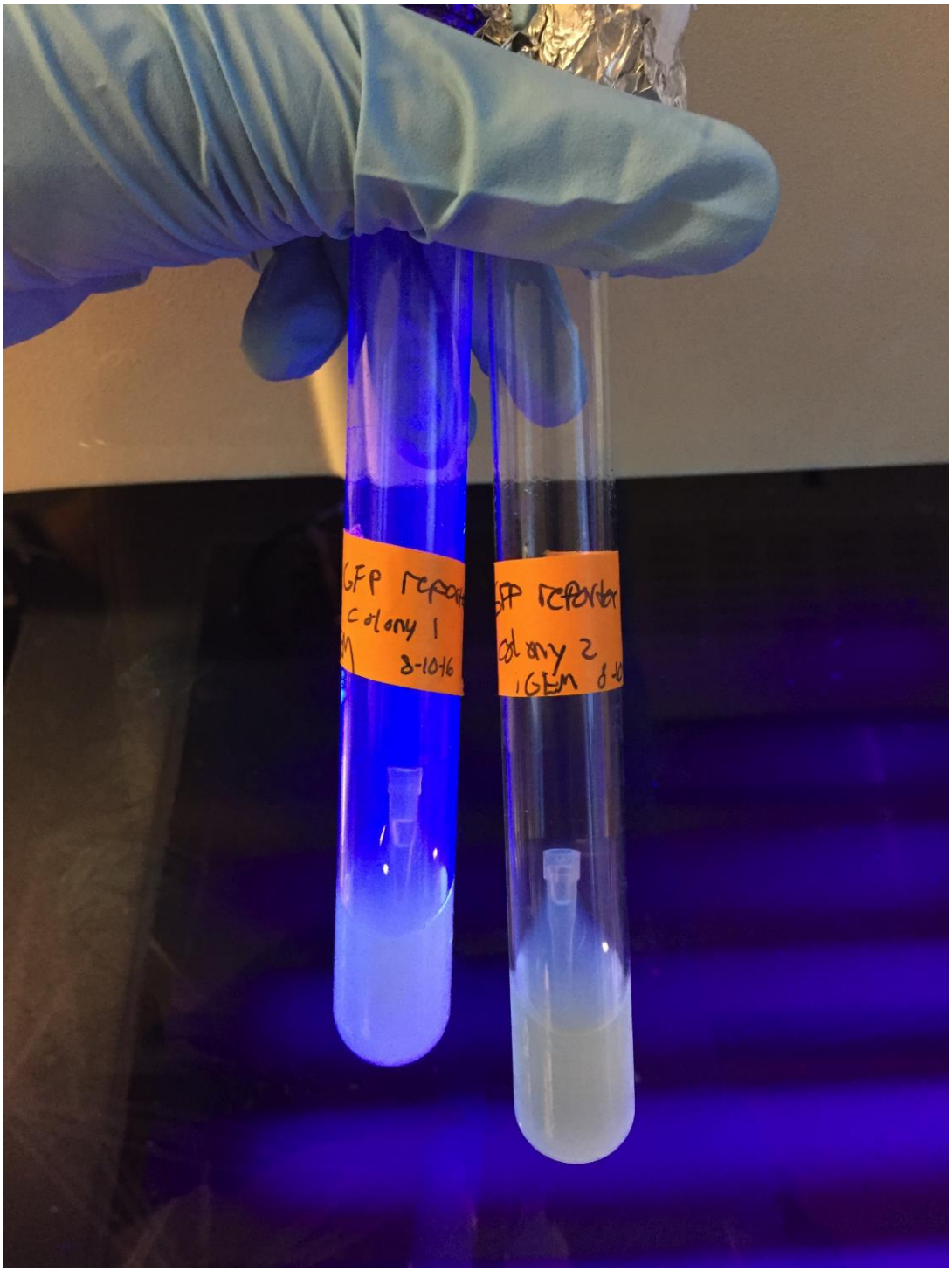
5. Grow your bacteria overnight at the appropriate temperature. Growth conditions, including copy number and growth temperature, can be found on your plasmid's information page. The next day you will be able to start an overnight culture for plasmid DNA prep the following day.

Note: So I only need to focus on steps 2-3 of the protocol for what we want to achieve regarding storing the bacteria in glycerol. As snap top tubes are not recommended, I asked Vasu and Walden from Zhao group what is the right tube. Turns out we need some type of tube that we don't have, so they gave us a packet. They are **orange capped** Corning plastic tubes about 2mL in volume and designed for -80degC. They are called Corning Cryogenic Vial.

What I did: I carried out steps 2 and 3, producing two tubes of glycerol stock for each colony 1 and 2 into the tubes I mentioned above. So we have a total of 2mL of glycerol stock for each of the two colonies that were inoculated yesterday night.

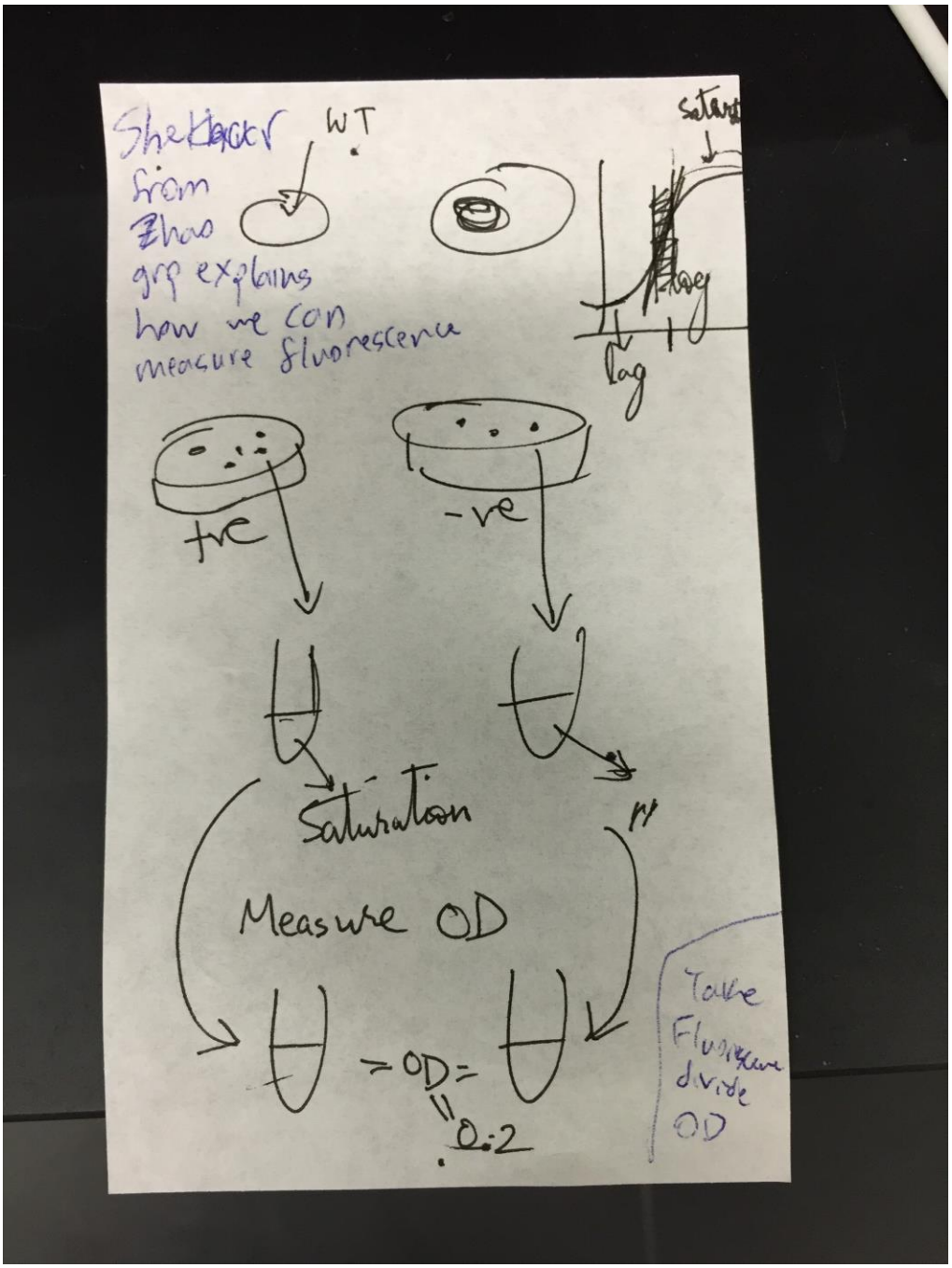
Miniprepping the bacteria transformed with the standard GFP ligation method (from the two colonies that were inoculated in liquid culture yesterday)

I carried out a miniprep very similar to what was done on 29 July 2016 - Performing miniprep on our Lycopene transformed cells (that were incubated overnight in LB tubes) with Viraat. Some notes
Step 1: I used whatever was liquid culture leftover after producing the glycerol stock and that amounted to about 3.5ml for each colony tube.



I had to ask the group how to get the UV light to be turned on in the gel-imager; it turns out I wasn't pulling the tray out far enough. When I did this, Ran Chao said that glass tubes might interfere as they filter out UV light.

But its funny, why does one tube "glow" blue while the other does not?



Aggregated remarks from Ran Chao and Shekhar (also from Zhao group) when I tried to qualitatively check for fluorescence this way is that its not very accurate. It might also be hard to see any fluorescence like this as the cells may not be expressing GFP. The best time to measure fluorescence is during the log phase for this reason, its more likely that cells are expressing GFP in log phase.

Shekhar explained to me how he does fluorescence measurement more precisely, and drew out the flow chart as on the right. No time to log down details as I gotta go, but the protocol is roughly similar to standard fluorescence imaging protocol. Use the same machine as our nanodrops concentration measurement, but read fluorescence using plates.What he refers to as "Wild Type" just simply means the equivalent strain of ecoli that does not have the GFP or whatever plasmid that we had transformed

into our ecoli strain (and not like real "wild type"). Fluorescence is relative measurement so the wild type is necessary to attenuate the effects of noise in measurement.

12 August 2016

Friday, 12 August 2016
2:43 PM

Goals for lab today are:
1. Run the plasmids that I miniprepped out from the two ligated GFP reporter construct colonies on a gel to verify identity. Need to do double digest followed by PCR purification first (elution with water), before I run on gel.
2. Take out the plates of Gibson GFP colonies from the incubator that I set up overnight, parafilm wrap them and store them in 4degC fridge.

For goal (1), I am following the procedure similar to 8 August 2016 notebook for a total of 20uL reaction, as we want to use a minimum of GFP plasmid DNA. Similar to that day, I am also using only 10uL of plasmid DNA here as I measured the concentrations of the Colony 1 to be 63.7ng/uL and Colony 2 to be 67.3ng/uL so using 10uL of each makes for about 637ng and 673ng respectively, which, when divided by the total volume of 20uL I guess makes for near enough to the desired concentration of 20ng/uL for the reaction to run. I ran the digestion for about 1hr 10mins at 37degC.

Followed the standard Qiagen PCR Purification protocol (copy and paste the protocol here), except with the following modifications: (a) Used water to elute the DNA instead of elution buffer (b) Used 30uL of water instead of 50uL to help increase the concentration of the eluted DNA and hopefully make the signal on the gel higher (because if too low, can't see anything…). From past experiences, generally the concentration of DNA we obtain after running purification protocol hasn't been as high as would be good for downstream steps. I created more DNA-dye mixture (total of 12uL from 10uL of DNA mixed with 2uL of loading dye) and also loaded a larger amount of DNA into the gel, putting 10uL into each well (I used the smallest wells possible, but now I think I should have used the larger wells…).

Ran the gel and here are the results:

<<insert gel photo here>>

Doesn't look good. Its like the restriction digestion didn't work at all!

For goal (2), my plate containing 0-fold dilution with SOC did contain a handful of small colonies separated far apart from one another! But the plate containing 1-fold dilution did not contain any colonies! Hmmm that means when the yield is low, its probably not a good idea to do any SOC dilution with the NEB protocol! I tried putting the 0-fold dilution colonies against the UV light from our gel imager to see if they fluoresced, but couldn't see any. Did this at 4pm.

15 August 2016

Monday, 15 August 2016
3:37 PM

Inoculated the 5 iGEM interlab study measurement kit constructs, together with the two Gibson constructs (one with 0-fold dilution, another with 1-fold dilution) with Mariam. Then I autoclaved all our dirty test tubes.

Tomorrow we'll carry out the plate reader protocol of the interlab study. Trying to understand the information about the protocol. I obtained one black-walled plate reader, but we might need more and we'll ask tmr.

The overall structure of the workflow is something like this: there's two phases, the calibration phase (where we do some measurements on chemicals provided to us, namely LUDOX and FITC, provided in the kit from iGEM), and the measurement phase (where we take measurements from the cells tranformed with the given iGEM parts). The calibration phase has two distinct steps, first to measure optical density, second to measure fluorescence. In the measurement phase, these two steps are carried out at the same time.

It seems like we'll need 3 plates? 2 for the two steps in calibration phase, 1 for the measurement phase?

16 August 2016

Tuesday, 16 August 2016
3:01 PM

Tasks completed

1. **Performed miniprep on the inoculated Gibson transformed cells (0-fold SOC dilution)**
    a. The 1-fold SOC dilution did not give any successful inoculations, likely because the transformation was not successfully (there were no colonies on the agar gel). I learnt from Caroline that the purpose of serial dilutions is actually reduce the amount of transformed cells that you introduce to your plate for cases when you transformation efficiencies are high. Turns out that our Gibson assembly yield was pretty low, so we had a very count of transformed cells to begin with, so doing serial dilutions will only make it harder to get successful transformation and therefore inoculation
2. **Carried out the entire calibration phase of the iGEM interlab study plate reader protocol with Caroline**

a. For the OD600 reference point protocol, we followed the instructions as provided by iGEM, but we did 2 kinds of OD read measurements under same setting of 2 second flashes with 10 milliseconds settle time: (1) Using just simple one read per well (2) Using a 4x4 filled circle sampling (the plate reader takes 16 small dots within each well and computes the average). The data is stored in our excel spreadsheet on the plate reader computer, and in general (2) gave much more variance in the data than (1)….

b. For the FITC fluorescence standard curve, we followed the protcol pretty closely, but when it came to the part of varying the settings to get different values, we mainly focussed on the ones that were accessible to us through the plate reader interface. We varied our Gain values, getting a healthy range from 50 to 110%. We are glad that we looked at gain, because the default settings for most of the pre-programmed protocols already on our machine used the choice of letting the machine calculate optimal gain each time. So that means that each time you take measurements, the machine is going to use a different gain value. But this would not be acceptable for our purposes of the interlab study as the settings that we are using for fluorescence calibration must be the same as that when we measure the interlab study cells. So we fixed the gain values at a few levels and took our measurements. We still kept our optimal values, but we'll take note of the actual gain value that the machine used so that if we choose them, we'll hardcode them into the plate reader. Data acquisition was easy as we had pre-programmed protocols (which included a "shaking" step that was necessary to ensure the liquid we added to the well was nicely spread out and not sticking to the walls or something) the plate reader would store all the information, machine settings and measured values, into an excel spreadsheet that we'll read from later to fill up the interlab study submission spreadsheet.

Somehow, the iGEM protocol wasn't clear, but only in retrospect did we realize that had we arranged the wells for the OD600 measurement in a horizontal direction, we could have used the same plate for both OD600 and FITC fluorescence measurements! But we ended up having to use one for each because we didn't and we did not have enough space for on the old plate used for OD600 to carry on with FITC. Our plates are the disposable type, once a well is used, there's no reusing it.

3. **Pelleted the interlab study cells that Mariam inoculated yesterday** We anticipated that we would do interlab measurements on them today. Unfortunately we were unable to use the cells because we missed out a simple requirement in the fluorescence calibration protocol of the interlab instructions (which we did as part 2(b) as that I described above) that preparation of the FITC stock solution for 2(b) requires an incubation time of 4hrs. Because we cannot proceed with the cell measurements without first completing calibration, this would mean that our inoculated interlab cells would be overgrown (they must not be incubated for more than 18hrs according to the interlab protocol) and unusable. So Caroline decided that we'll pellet up those cells and freeze them in -20degC, a common wetlab practice when you have DNA that you want to archive, but do not want to bother with extracting out anytime soon. The procedure is to just cetnrifuge the liquid culture in plastic tubes, then pour away the supernatant and store in -20degC in the tubes. Our plan is to then do the same inoculation again tomorrow evening, so that we can take out these cells in the morning of Thursday and carry out the procedure uninterrupted (we need to make measurements in 1 hr intervals, but on Wed we have the group meeting). Oh, and we for each cell that we inoculate, we must inoculate another copy of it, something which we missed out when we did it on Tuesday.

17 August 2016

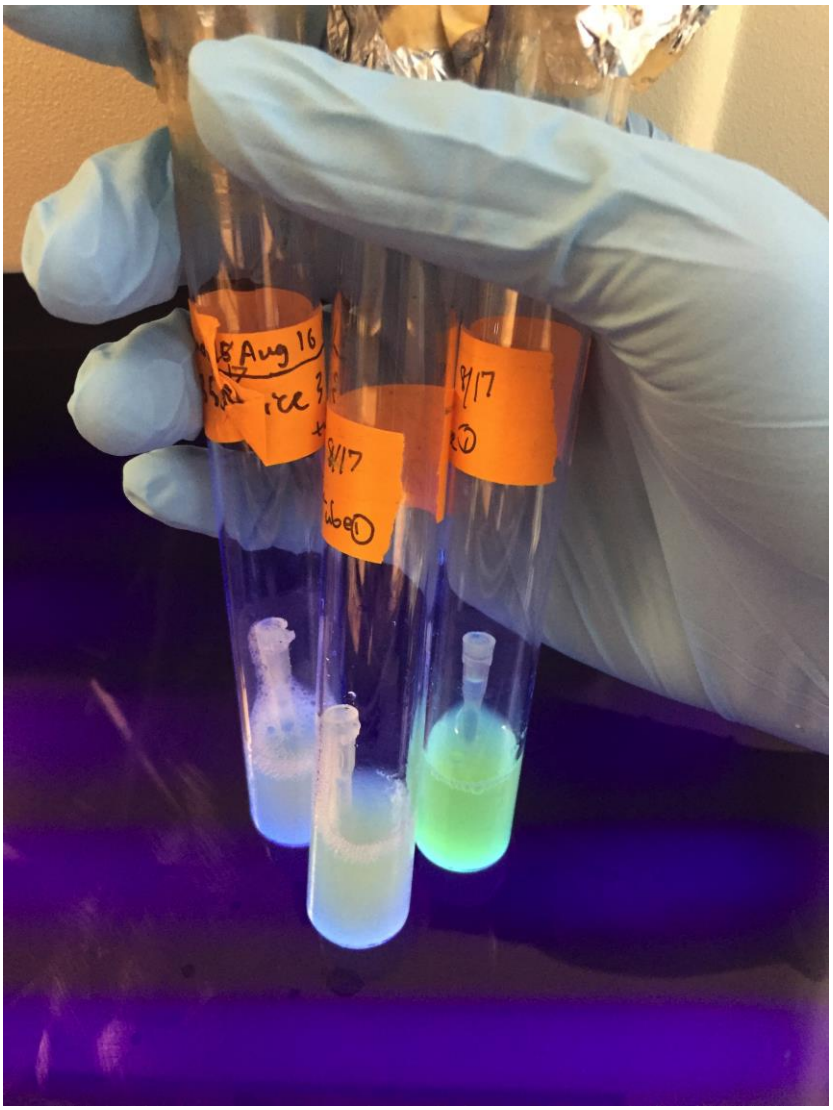
Thursday, 18 August 2016
11:35 PM

Backtrack update - created this notebook after the day itself (see the date created).

Basically nothing much to write for lab work on Wed 17 August… Mainly we tried to submit our parts for sequencing but when doing so realized that we had too little DNA left to actually submit, and that motivated us to redo a transformation that day and inoculation on 18 August to accumulate more DNA for submission to our sequencing center in Madigan Lab.

18 August 2016

Thursday, 18 August 2016
10:23 AM



Main goal for today is to complete the interlab study cell measurement protocol

Started executing the interlab study cell measurement protocol. First took out the inoculated interlab study parts from the incubator at about 825am which is just about 18 hours of total incubation time (since we started incubating them at 225pm yesterday). So we did not go beyond 18hrs, and are at the maximum incubation time allowed by the interlab protocol. Device 1 was fluorescing as I took it out! See the photo on the right.

Immediately placed these tubes on ice to pause the growth.

First step of the protocol requires us to use Measured OD600 (also known as Absorbance 600) using Thermo Scientific GENESYS 10S UV-Vis spectrophotometer machine. Followed their documentation on pg 44 that states:

Measuring a Blank

To measure a blank

1. Place the blank in the cell holder.
   If a 6-Position Cell Changer is installed, place the blank in the B position.
2. To enter an absorbance or transmittance value for the blank, press a number key and enter the desired value in the **Entry** field.
3. Press **Measure Blank**.

Measuring Samples

If a 6-Position Cell Changer is installed, place the samples in the cell positions and press the corresponding cell position button to move the cell holder to the measuring position. The absorbance (ABS) or percent transmittance (%T) measurement appears on the display.

I used those small square curvettes, making sure to place them in to photometer with the clear plastic side exposed to the hole through which photon measurement could be taken.

So I have the results below. Because the spectrophotometer could only take at most 5 tubes for measurement at any one time, I had 2 rounds of measurement.
Did not do step 2 as I wanted the machine to set the blank to be value 0. Blanking the spectrophotometer is very similar to a zeroing on the weighing scale.

	Measurement Round	Position in Spectrophotometer	OD600 or Absorbance
Positive Control Culture 1	2	1	1.655
Positive Control Culture 2	1	1	1.669
Negative Control Culture 1	1	2	1.682
Negative Control Culture 2	1	3	1.700

Device 1 Culture 1	2	5	1.573
Device 1 Culture 2	2	4	1.575
Device 2 Culture 1	1	4	1.667
Device 2 Culture 2	1	5	1.703
Device 3 Culture 1	2	3	1.748
Device 3 Culture 2	2	2	1.753

I felt that the measurements for Device 1 cultures 1 and 2 was problematic, because the OD600 reading from the machine kept on decreasing, so I did not know when to take the reading. I asked Sam Hamedi for help, and he suggested that I could just ignore the 3rd decimal place. The reason for the drop in value is because of cells sinking to the bottom of the curvette (pelleting)

Entering the OD600 values into an excel spreadsheet provided by iGEM HQ, the formulas encoded in the spreadsheet could calculate the required dilution for us. We ran out of CM so they had to make some more before doing the dilution. Here is the spreadsheet from iGEM below, all filled up:

target Abs600		**0.02**	
target volume (mL)		**10**	
sample	Abs600 reading	volume of preloading culture	volume of preloading media
positive control culture 1	1.655	0.120845921	9.879154079
positive control culture 2	1.669	0.119832235	9.880167765
negative control culture 1	1.682	0.118906064	9.881093936
negative control culture 2	1.7	0.117647059	9.882352941
device 1 culture 1	1.573	0.127145582	9.872854418
device 1 culture 2	1.575	0.126984127	9.873015873
device 2 culture 1	1.667	0.119976005	9.880023995

device 2 culture 2	1.703	0.117439812	9.882560188
device 3 culture 1	1.748	0.114416476	9.885583524
device 3 culture 2	1.753	0.114090131	9.885909869
media+chl	0	#DIV/0!	#DIV/0!

The undefined values for media+chl makes sense because its abs600 reading was already at 0! Basically this value is just meant for us to be a baseline to know where our other cultures are at so that we can dilute correctly (since our cultures are using the media+chl for a medium).
Then we carried on with the entire protocol, pipetting out the diluted cultures into wells on a plate at regular 1 hour intervals for 6 hours. The plate was placed on ice as instructed by the interlab study so that we stop the growth of the cells that we pipette out onto the corresponding wells on the plate at each timepoint (therefore maintaining the fidelity of the measurement). We willed fill out the entire plate and tookall the measurements from 0-6 hrs at one go.

So we ended the day with taking the measurements on the plate. Turns out the values for fluorescence look really good (they show an increase over time, while we don't really know what to expect from the interlab kit, and although we can look at the parts we were provided, iGEM didn't explicitly state what would make for a good result) but OD did not look too good (there was some fluctuation, no constant trend). We guess that maybe some cells may have died but then grew again, it could be that we did not keep accumulating new cells! We took a few measurements, but one that looks really good, we added the keyword "GOOD" into the name and mailed all the results to ourselves. Hmmm there was one timepoint, I think it was 4 or 5, where we made a trivial mistake of pipetting culture 2 of device 1 into the well meant for culture 1 of device 1. We tried to undo that, and this shouldn’t affect too much too since it’s the same device, but could this have affected the OD values? :-/

Other tasks and notes:

- We poured new LB+CM plates. Best to always leave them to cool down after pouring with the LB side down, even after the LB has solidified. Flip them over too early and the LB could drop from the top side down and render the plate unusable.
- The Gibson and Ligated GFP colony that we transformed yesterday grew a lot, possibly overgrown. The whole plate was just covered in dots of colonies! Might not be too good of a thing. Caroline was skeptical of whether the right cells actually grew. We inoculated both of them in liquid culture in falcon tubes together with an additional negative control taken right from the interlab study kit (yes, we still have a few negative control colonies from our interlab study kit transformations!) for good measure. Tomorrow we'll be miniprepping those to accumulate enough DNA to send for sequencing!

28 August 2016

Sunday, 28 August 2016
4:05 PM

Filling up the official iGEM interlab study spreadsheet with our data from our plate reader expts following the interlab protocol. The data is due on Friday, 2nd Sep. Some notes:

- The interlab protocol requires us to fill more wells with LB+CM solution during the measurement phase of the interlab experiment, more than what we will actually need to key in into the spreadsheet (under the sheet "cell measurement")! So which wells did I exclude? Bascially all of row H from our plate readings (so I only filled in the data of columns 11 and 12 for LB_CM fluorescence and OD readings).

- Decided to use our GOOD data that was taken at a gain of 87%. The data for that was the most consistent and sensible among the measurements we recorded. And yes, we did follow this value of gain of 87% both during our FITC serial dilution calibration phase, and also during our growing cells device measurement phase!

- For our LUDOX OD600 reference measurement, we did two readings with different configurations, (1) single read per well (2) multiple reads per well (with a 4x4 circle averaged within a single well). But the data for (2) turned out to be way more inconsistent than (1) (see the table below) so we decided to use the data for (1)

Single Read	LUDOX	H2O
replicate 1	0.0449	0.038
replicate 2	0.042	0.0344
replicate 3	0.0487	0.0357
replicate 4	0.0487	0.0374

Multiple Reads	LUDOX	H2O
replicate 1	0.0539	0.06408
replicate 2	0.0462	0.0371
replicate 3	0.0444	0.0359
replicate 4	0.0604	0.0389

Lab work done on 16 Sep 2016

Friday, 16 September 2016
5:43 PM

Goals were to carry on from where Jonathan and Caroline left off, and to progress towards successful assembly of our promoter-GFP plasmid constructs. The goalsare:

- Do PCR purification on:
  - the promoter-primers mixtures that Caroline did to PCR amplify additional promoters
  - the linear backbone plasmid which Jonathan also did PCR on to amplify for more plasmid backbone

- Digest these parts following the http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones#Digest
- Ligate them to get the construct

We were only able to complete PCR purification and the digestion of the linearized plasmid backbone. Do we actually have to digest our promoter parts? Wait hmm… these promoter parts were PCRed out using new primers which contain Biobrick prefix and suffix (see our promoter spreadsheet) since they used primers 30-31, 32-33, 34-35, so does that mean that they were already PCRed out with these sites? So we weren't sure and thus didn't carry it out. Stored them in tubes with date labelled

Mariam and I did the PCR purification in pretty much standard Qiagen protocol, main quirk is that we elute with 30uL of water. Since the linear backbone was PCRed in 2 batches by Jojo, we carried out our digestions on these two batches as separate tubes in parallel. One of the tubes had a concentration of 122.2ng/uL and the other had 161.5ng/uL according to nanodrops measurement.

As for linear backbone digestion, we followed iGEM website but with the following distinctions

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)
  - ~~5 ul NEB Buffer 2~~ [5ul Cutsmart Buffer]
  - 0.5 ul BSA
  - 0.5 ul EcoRI-HF
  - ~~0.5 ul PstI~~ [0.5ul PstI-HF]
  - 0.5 ul DpnI (Used to digest any template DNA from production)
  - 18 ul dH20
    - *** we had leftovers of this enzyme master mix which we are storing in the Misc DNA box, in tube labelled with date
- Digest Plasmid Backbone
  - ~~Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)~~ Add 1uL from both tubes. As their concentrations were high enough, we figure we'll just add 1uL (with us exceeding 100ng slightly) as adding any lower than that could introduce measurement errors.
  - Add 4 ul of Enzyme Master Mix
  - Digest 37C/30 min~~, heat kill 80C/20 min~~ Cannot heatkill the HF enzymes, so I followed up the digestion with a PCR purification in a similar manner as what I recorded on 8 August 2016

Completed the PCR purification, and labelled the tubes with date on them. Called the tube which was descended from the 161.5ng/ul conc batch of linear undigested PCR amplified backbone (upon PCR purification) as batch 2 and the one which was from the 122.2ng/uL conc as batch 1.

20 September 2016

Tuesday, 20 September 2016
1:55 PM

Today just carry on the work towards our promoter construct. Tasks left to do in order:

- Digest the promoter sequences (completed)
  - Also redo digestion for linear plasmid backbone
- Ligate them with the digested backbone (not yet)

Starting out, I realized that the total volume of reaction mixture that we used to digest the backbone yesterday added up to < 8ul (although the mass of backbone used was sufficient) which would imply that the reaction won't turn out well. I decided to redo the backbone digestion, this time adhering to the volume ratio of 4uL enzyme master mix with 4uL backbone/promoter part.

So first thing I had to do was to produce a diluted sample of each part to be digested. I did nanodrops measurement and calculated the volumes on our notebook:

From 30 — 106 ng/µl

32 — 60.2 ng/µl

34 — 41.1 ng/µl

~~All~~ Goal is to dilute to 25

34 $\frac{41.1}{25} = 1.644 \approx$ ~~1.7~~

32 $\frac{60.2}{25} = 2.408 \approx 2.4$

30 $\frac{106}{25} = 4.24 \approx$ ~~4.25~~

≈ 4.2

Mix 10 µl of 34 with

16 µl of dH_2O

$\frac{106}{4.}$

228

Batch 2

161.5 ng/ul

Batch 1

122.2 ng/ul

20 Sep

$$\frac{161.5}{25} = 6.46$$

$$\frac{122.2}{25} = 4.88$$

Digested
three
~~[illegible]~~
promote
Since
each of
a back
we n
to

mix 10 ul of batch 2 with
64.6 ul of ~~[illegible]~~ ddH2O

10 ul of batch 1 with

As a result of the dilutions, I have some leftover samples of diluted parts (at 25ng/uL) which I stored in labelled tubes in our Misc DNA box.

Next I carried out the digestion using the standard iGEM protocol with slight modification (basically the same to yesterday's except that the volumes are now being adhered to). Followed up with PCR purfication to remove HF enzymes and kept the tubes in freezer.

Was not feeling well enough to continue through with ligation.

Other notes:
*Our promoters this time (generated from PCR by Caroline using primers with Biobricks prefix and suffix ends) while have the biobrick ends, still need to be digested
*We are not ligating with any GFP constructs, because we are just going to clone these 3 promoters and send to iGEM HQ (executing backup plan now to just produce some parts and submit to HQ, at least we want to have these parts at hand first in case GFP does not work out in time), also our GFP parts are still in shipment, not yet arrived

30 Sep 2016

Friday, 30 September 2016
7:28 PM

Completed the following with Mariam

- Running the gel on our 6 promoter parts (36, 38, 40, 42, 44, 46) and then extracting them out following Qiagen standard gel extraction protocol. Only thing did differently from protocol is to elute with unusually small amount of buffer EB (just 20uL). This can hopefully give us a high yield. In fact, the reason why we have been getting such low yields in all our previous purifications could be because we were eluting with water.

- Did digestion on 2 batches of linearized plasmid backbones which were obtained from PCR. Following iGEM protocol it says that we can do digestion immediately without first purifying the mixture obtained after PCR, so we just digested. The digestion mixture remains to be purified. Added 4uL of each backbone-PCR mix to 4uL of enzyme master mix. The backbone-PCR mixture was at 488ng/UL for batch 1, and 484.3ng/uL for batch 2.

  It remains for these digestion mixtures to be purified next, before ligation step.

6 October 2016

Thursday, 6 October 2016
3:28 PM

Just performed miniprep on 6 tubes of inoculated cells. The tubes were labelled Colony 1 Tubes A and B, Colony 2 Tubes A and B, and Colony 3 Tubes A and B. Followed Qiagen standard protocol as described on 3rd August 2016, with elution using 30uL ddH2O.

I only saw Caroline's message after I was done, that I should've used NF (nuclease free) H2O :(

12 October 2016

Wednesday, 12 October 2016
6:47 PM

Purpose of lab today was to ligate our promoters 36,38,40,42,44,46 with the backbone and then transform them into dh5alpha competent cells.

These promoters have been run on, and extracted from the gel.

Carried out ligation following NEB protocol on https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202 but with modifications (read below)

Protocol

1. Set up the following reaction in a microcentrifuge tube on ice.
(T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)*	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours*(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
5. Heat inactivate at 65°C for 10 minutes.
6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

But the amounts of added vector DNA and insert DNA have been modified to suit our concentrations and volumes.

Caroline suggested we add 25ng of Backbone with 20ng of promoter.

The concetration of our backbone was measured at 13.6ng/ul. I carried out nanodrops concentration measurement of insert (promoter DNA) gave the following in units of ng/ul:

Prom 36	Prom 38	Prom 40	Prom 42	Prom 44	Prom 46
49.3	26.5	14.3	38	20.8	39.7

Since Prom 36,38,42,46 were at relatively high conc, it taking 20ng from them would require very small volumes that are very near lower bound of our pipettes, so I diluted them by 3x, 2x, 2x, 2x respectively

So that meant I had to use 1.84uL of backbone with the following volumes of promoters:

Prom 36	Prom 38	Prom 40	Prom 42	Prom 44	Prom 46
1.217	1.5094	1.3986	1.0526	0.9615	1.0076

I mixed these volumes of promoters, backbones, and ligation enzyme and buffer and topped up with Nuclease free H20 accordingly to 20uL total.

Modification to incubation protocol from NEB version: I just incubated at room temperature for 30mins, but then followed heat inactivation at 65degC for 10mins using thermocycler.

After ligation, I followed Jonathan's protocol (which he used yesterday to transform Oxford parts) to transform the promoters into competent cells

Transformation Protocol:

1. Remember you need to thaw competent cells.
2. Dh5alpha cells for about 20 minutes on ice
3. Warm agar plates in 37C incubator.
4. Place 50µL of competent cells in 1.5 mL microcentrifuge tube.
5. Place 5µL plasmids in 1.5 mL microcentrifuge tube
6. Ice bath for 30 minutes
7. Heat shock (45 seconds) in 42 degree water bath
8. In the micro centrifuge, pipet 500µL of LB broth
9. Place tubes in flask
10. Secure to spinning incubator
11. Incubate for 45 minutes (shaking one, 37 degree)
12. Take 200µl (small amount)
13. Streak chlorophenicol plates using balls
14. Incubate overnight in the top part of the spinning incubator (37C)

Experiment is complete. I'm tired. I started at 5pm and ended now at 915pm. Its time to go home….